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RESEARCH ARTICLE

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Comparative cytogenetics of *Physalaemus albifrons* and *Physalaemus cuvieri* species groups (Anura, Leptodactylidae)

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Abstract

Recently, *Physalaemus albifrons* (Spix, 1824) was relocated from the *P. cuvieri* group to the same group as *P. biligonigerus* (Cope, 1861), *P. marmoratus* (Reinhardt & Lütken, 1862) and *P. santafecinus* Barrio, 1965. To contribute to the analysis of this proposition, we studied the karyotypes of *P. albifrons*, *P. santafecinus* and three species of the *P. cuvieri* group. The karyotype of *P. santafecinus* was found to be very similar to those of *P. biligonigerus* and *P. marmoratus*, which were previously described. A remarkable characteristic that these three species share is a conspicuous C-band that extends from the pericentromeric region almost to the telomere in the short arm of chromosome 3. This characteristic is not present in the *P. albifrons* karyotype and could be a synapomorphy of *P. biligonigerus*, *P. marmoratus* and *P. santafecinus*. The karyotype of *P. santafecinus* is also similar to those of *P. marmoratus* and *P. biligonigerus* owing to the presence of several terminal C-bands and the distal localization of the NOR in a small metacentric chromosome. In contrast, the *P. albifrons* karyotype has no terminal C-bands and its NOR is located intersti-

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tially in the long arm of submetacentric chromosome 8. The NOR-bearing chromosome of *P. albifrons* very closely resembles those found in *P. albonotatus* (Steindachner, 1864), *P. cuqui* Lobo, 1993 and some populations of *P. cuvieri* Fitzinger, 1826. Additionally, the *P. albifrons* karyotype has an interstitial C-band in chromosome 5 that has been exclusively observed in species of the *P. cuvieri* group. Therefore, we were not able to identify any chromosomal feature that supports the reallocation of *P. albifrons*.

Keywords
Chromosome, NOR, C-banding, heterochromatin, *Physalaemus*

Introduction

Currently, the genus *Physalaemus* Fitzinger, 1826 is classified in the subfamily Leiuperinae Bonaparte, 1850 in the family Leptodatylidae Werner, 1896 (Pyron and Wiens 2011) and is composed of 46 species (Faivovich et al. 2012, Frost 2013). A detailed phylogenetic analysis of the species of *Physalaemus* is not yet available but some supraspecific groupings have been proposed. Lynch (1970) recognized four species groups: *P. pustulosus*, *P. biligonigerus*, *P. cuvieri* and *P. signifier*, which was followed until recently. Based on a phenetic analysis of morphometric characters, Nascimento et al. (2005) resurrected *Engystomops* Jiménez de la Espada, 1872 to include the species previously allocated to the *P. pustulosus* group (*sensu* Lynch, 1970), resurrected *Eupemphix* Steindachner,1863 for the single species *E. nattereri* Steindachner, 1863 (included in the *P. biligonigerus* group by Lynch, 1970) and recognized seven species groups of *Physalaemus*: *P. albifrons*, *P. cuvieri*, *P. deimaticus, P. gracilis, P. henselii*, *P. olfersii* and *P. signifer*. Because *Eupemphix* was paraphyletic with respect to *Physalaemus* in phylogenetic analyses that included eight (Pyron and Wiens 2011) and five (Faivovich et al. 2012) species of *Physalaemus*, Faivovich et al. (2012) proposed that *Eupemphix* is a junior synonym of *Physalaemus*, but did not allocate *P. nattereri* to any species group. The monophyly of each of the seven species groups of *Physalaemus* proposed by Nascimento et al. (2005) remains to be tested and possible synapomorphies of these groups are still to be recognized (see comments in Borteiro and Kolenc 2007, Tomatis et al. 2009, Vera Candioti et al. 2011).

According to the taxonomic proposal of Nascimento et al. (2005), *P. albifrons* (Spix, 1824) was removed from the *P. cuvieri* group (*sensu* Lynch 1970) and grouped together with *P. biligonigerus* (Cope, 1861), *P. marmoratus* (Reinhardt and Lütken, 1862) and *P. santafecinus* Barrio, 1965, three species that were previously allocated to the *P. biligonigerus* group proposed by Lynch (1970). Interestingly, Lobo (1996) indicated that the species of the *P. biligonigerus* group (*sensu* Lynch 1970; that included *P. marmoratus*, as *P. fuscomaculatus*) shared shovel-shaped metatarsal tubercles with *P. albifrons*. Otherwise, Vera Candioti et al. (2011) argued that larval oral morphology does not support the reallocation of *P. albifrons* proposed by Nascimento et al (2005), because the larval oral configuration of *P. albifrons* is almost identical to that of members of the *P. cuvieri* species group and differs from that of *P. biligonigerus*, *P. santafecinus* and probably *P. marmoratus*.

Detailed descriptions of the karyotypes of *P. biligonigerus* and *P. marmoratus* (as *P. fuscomaculatus*), which included the identification of the nucleolus organizer regions (NOR) and heterochromatic sites, were already provided (Amaral et al. 2000, Silva et al. 2000). On the other hand, only the chromosome number and morphology are known for *P. albifrons* (Denaro 1972), and no chromosomal data are available for *P. santafecinus*.

In the present work, we present a detailed characterization of the karyotype of *P. albifrons*, describe the karyotype of *P. santafecinus* and extend the cytogenetic analyses of the *P. cuvieri* group in order to better characterize the group from which *P. albifrons* was removed. Our aim is to provide additional evidence that could be used to compare the *P. albifrons* and P. *cuvieri* species groups.

Materials and methods

Specimens of *P. albifrons*, *P. santafecinus*, *P. albonotatus* (Steindachner, 1864), *P. centralis* Bokermann, 1962 and *P. cuqui* Lobo, 1993 from different localities in Brazil and Argentina were analyzed. For an unequivocal identification of the species, both morphological and acoustic characteristics were utilized. Each specimen's locality and voucher number in the scientific collection where it was deposited are provided in Table 1.

Metaphase chromosome spreads were obtained from cell suspensions of the intestine and testes of animals pre-treated with colchicine (2%) for at least 4 hours (according to Schmid et al. 2010, or adapted from King and Rofe 1976). Prior to the removal of the intestine and testes, the animals were deeply anesthetized with lidocaine gel 2%. Chromosomes were conventionally stained with 10% Giemsa and sequentially submitted to C-banding (King 1980) and silver staining by the Ag-NOR method (Howell and Black 1980) or to fluorescence *in situ* hybridization (FISH) (Viegas-Péquinot 1992) with the rDNA probe HM 123 (Meunier-Rotival et al. 1979). C-banded metaphases from *P. albifrons* were also stained with DAPI (0.5 mg/mL). For each species, at least 10 metaphases that were submitted to each technique were analyzed. Morphometric analyses were done using the MICROMEASURE v3.3 software (Reeves and Tear 2000). The chromosomes were classified according to the criteria proposed by Green and Sessions (1991).

Results

All of the analyzed individuals had a diploid complement of 22 chromosomes. By comparing all of the karyotypes of *Physalaemus* to each other, we noted a high interspecific similarity for the first seven chromosome pairs, and the homeology of these chromosomes could be inferred. Therefore, in each karyotype presented here, these chromosomes were ordered in such a way that their numbers could reflect these homology hypotheses even when their sizes suggested a different numbering. However, the smallest chromosomes (pairs 8–11) varied significantly among the species analyzed, and were numbered only

Table 1. Locality, voucher number and chromosome location of NORs and C-bands of the analyzed specimens. Abbreviations: CH – Chaco province; CT – Corrientes province; MA – Maranhão state; MT - Mato Grosso state; SA – Salta province; SP - São Paulo state; TO - Tocantins state; BC - Departamento de Biologia Celular da UNICAMP, Campinas, SP, Brazil; LGE - Laboratorio de Genética Evolutiva, Facultad de Ciencias Exactas Químicas y Naturales, Universidad Nacional de Misiones, Posadas, Misiones, Argentina; MNRJ - Museu Nacional do Rio de Janeiro, RJ, Brazil; UFMT - Universidade Federal do Mato Grosso, MT, Brazil; ZUEC - Museu de História Natural, Universidade Estadual de Campinas, Campinas, SP, Brazil; p: short arm; q: long arm; int: interstitial; per: pericentromeric; 3cen-per: centromeric band that extend to the short arm. * only one chromosome of the pair. **In the ZUEC 13696 specimen, an additional terminal C-band is present in 2q (see text for details).

Species	Locality	Specimens	NOR locations	Principal non-centromeric C-bands
P. albifrons	Barreirinhas, MA, Brazil	**7** ♂ (MNRJ 24228, 24230, 24232, ZUEC 12361–3, 17925), **1**♀ (MNRJ 24227)	8q	3cen-per/5p int/8p per/ 9p per
P. albonotatus	Lambari D´Oeste, MT, Brazil (57.4°W, 16.4°S)	**6** ♂ (UFMT 4462, 4466, 4469–72), **1**♀ (UFMT 4465)	8q/9p/9q	2q int/3cen-per/5 int/
P. centralis	Palestina, SP, Brazil (49.2°W, 20.2°S)	**5** ♂ (ZUEC 13689–90, 13692, 3694, 13696)	9q per	2q int**/3cen-per/5p int/ 8q int/9q int/10p per
	Porto Nacional, TO, Brazil (48.6°W, 10.4°S)	**3** ♂ (ZUEC 13373, 13375, 13380)	9q per	2q int/3cen-per/5p int/ 8q int/9q int/10p per
P. cuqui	Near to Rio Piedras, Iruya, SA, Argentina (22°56'S; 64°39'W)	**1**♀ (LGE 6567)	3p*/8q/9p/9q	2q int/3cen-per/5p int
	Taco Pozo, CH, Argentina (25°34'S, 63°09'W)	**2** ♂ (LGE 1635–6)	8q/9p/9q	2q int/3cen-per/5p int
	Aguas Blancas, SA, Argentina (22°43'S, 64°22'W)	**1**♀ (LGE 6568)	8q/9p/9q	-
	Metán, SA, Argentina (25°06'S, 65°03'W)	**1**♀ (LGE 6569)	8q*/9p/9q	2q int/3cen-per/5p int
	Pichanal, SA, Argentina (25°24'S,64°09'W)	**1**♂ (LGE 6570)	8q/9p/9q	2q int/3cen-per/5p int
P. santafecinus	Ituzaingó, CT, Argentina (27°31'S, 56°40'W	**6** ♂ (LGE 077–8, 083–4, 087–8)	9q	1p per/1q int/2p per/3p/8p per/7q per/terminal in all chromosomes

by chromosome size. A detailed description of the karyotype of each species is presented below and the Appendix (Additional file 1) present all the karyotypes arranged together. Table 1 summarizes the data on NORs and non-centromeric C-bands.

Physalaemus albifrons

The *P. albifrons* chromosomes were classified as metacentric (pairs 1, 2, 5, 6, 9 and 11), submetacentric (pairs 4, 7, 8 and 10) or subtelocentric (pair 3, which is at the threshold between submetacentric and subtelocentric classifications) (Fig. 1a; Table 2). C-banding followed by DAPI staining detected all of the centromeric regions and an

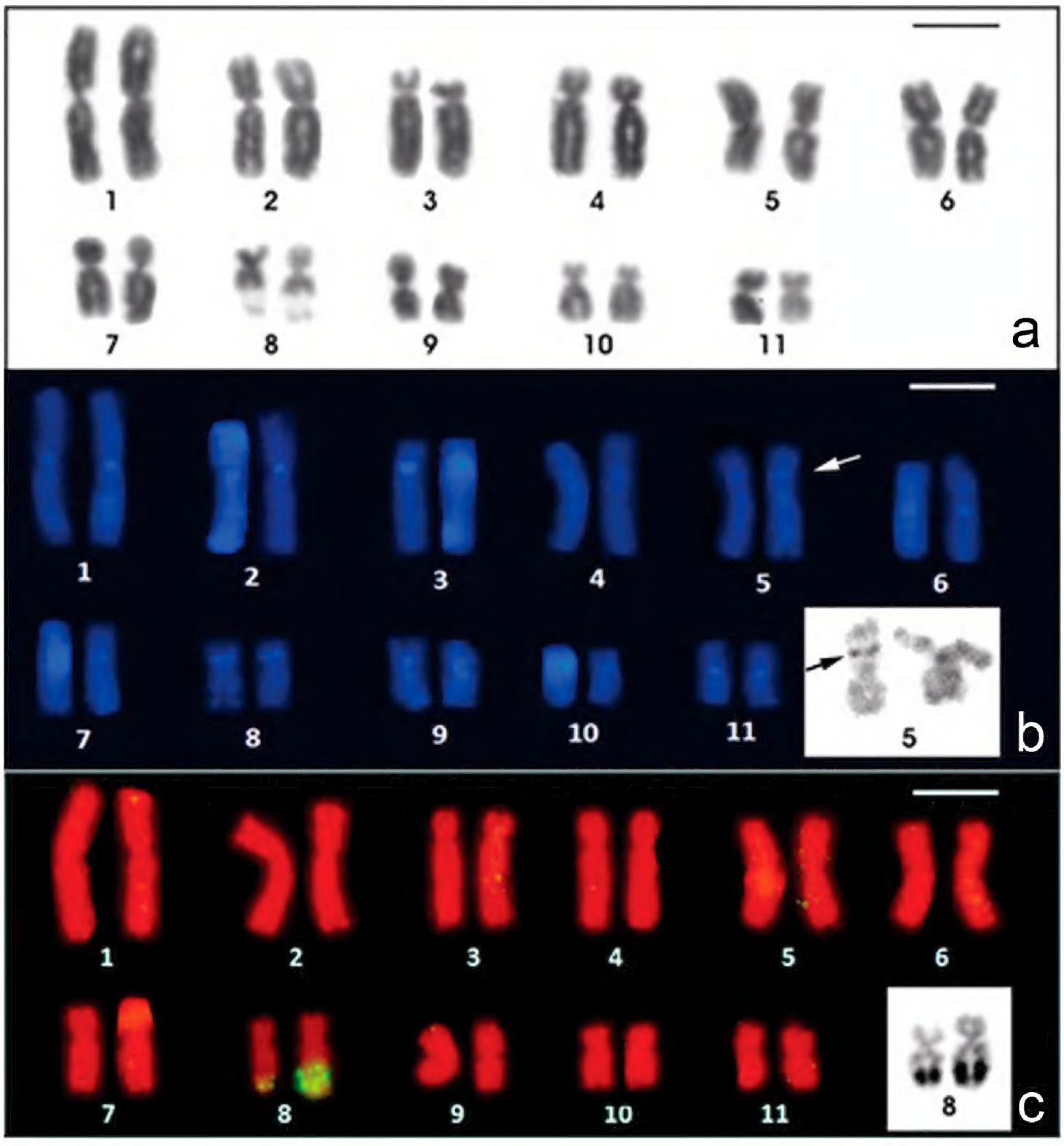

Figure 1. Karyotypes of *P. albifrons* after Giemsa-staining (**a**) C-banding followed by DAPI-staining (**b**) and in situ hybridization with a nucleolar rDNA probe (**c**). In **b** the inset shows the C-banded chromosome pair 5 stained with Giemsa; in **c** the inset shows the NOR-bearing chromosome pair 8 after silver staining. Arrows in **b** point the interstitial C-band in 5p. Bar=10mm.

interstitial heterochromatic band in the short arm of chromosome 5 as well as pericentromeric bands in the short arm of chromosomes 8 and 9 (Fig. 1b). The Giemsa stained C-banded metaphases showed this same pattern, but after DAPI staining, the bands could be more easily visualized. Chromosomes 3 and 4 were very similar, but chromosome 3 had a slightly smaller centromeric index and a strong centromeric C-band, which extended to the short arm (Fig. 1b; Table 2).

The NORs were located distally in the long arm of chromosome 8 (Fig. 1c) and coincided with the secondary constrictions that were observed in Giemsa-stained meta-

Table 2. Morphometric parameters of the *P. albifrons*, *P. albonotatus*, *P. centralis*, *P. cuqui* and *P. santafecinus* karyotypes. The measurements were based on 10 metaphases of each species. CN: chromosome number; CI: centromeric index; SD: standard deviation; RL: relative length. CC: chromosome classification; m: metacentric; sm: submetacentric; st: subtelocentric. *[1]Chromosomes were numbered in order to reflect our hypotheses of homeology for the *Physalaemus* chromosomes even when their sizes suggested a different numbering. *[2]Value at the threshold between submetacentric and subtelocentric classifications.

P. albifrons											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.47 ± 0.03	0.40 ± 0.04	0.24 ± 0.02	0.29 ± 0.02	0.46 ± 0.02	0.44 ± 0.04	0.36 ± 0.02	0.33 ± 0.04	0.43 ± 0.04	0.28 ± 0.04	0.45 ± 0.04
RL(%)	14.68	12.15	10.06*[1]	10.64*[1]	9.68	9.43	8.27	7.17	6.76	5.94	5.88
CC	m	m	st*	sm	m	m	sm	sm	m	sm	m
P. albonotatus											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.46 ± 0.03	0.45 ± 0.04	0.24 ± 0.02	0.33 ± 0.02	0.46 ± 0.03	0.43 ± 0.04	0.36 ± 0.07	0.39 ± 0.04	0.44 ± 0.03	0.42 ± 0.03	0.46 ± 0.03
RL(%)	13.87	12.18	10.00*[1]	10.42*[1]	9.61	9.48	8.31	7.32	7.05	5.98	5.78
CC	m	m	st*	sm	m	m	sm	m	m	m	m
P. centralis											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.47 ± 0.01	0.39 ± 0.01	0.26 ± 0.02	0.30 ± 0.03	0.46 ± 0.03	0.43 ± 0.03	0.35 ± 0.03	0.42 ± 0.05	0.45 ± 0.01	0.40 ± 0.02	0.40 ± 0.04
RL(%)	13.82	12.24	10.07*[1]	10.26*[1]	10.03	9.36	7.99	7.27	7.12	6.31	5.52
CC	m	m	sm	sm	m	m	sm	m	m	m	m
P. cuqui											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.47 ± 0.02	0.41 ± 0.03	0.24 ± 0.05	0.30 ± 0.03	0.44 ± 0.03	0.41 ± 0.02	0.34 ± 0.03	0.42 ± 0.01	0.42 ± 0.06	0.38 ± 0.01	0.43 ± 0.03
RL(%)	14.53	13.57	10.0*[1]	10.36*[1]	9.93	9.49	8.39	7.08	6.07	5.40	5.19
CC	m	m	st*	sm	m	m	sm	m	m	m	m
P. santafecinus											
CN	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
CI ± SD	0.46 ± 0.02	0.40 ± 0.01	0.39 ± 0.02	0.27 ± 0.02	0.46 ± 0.02	0.43 ± 0.01	0.32 ± 0.02	0.39 ± 0.03	0.47 ± 0.01	0.43 ± 0.03	0.43 ± 0.04
RL(%)	14.11	13.21	12.34	10.88	10.27	9.67	8.60	5.68	5.41	5.35	4.47
CC	m	m	m	sm	m	m	sm	m	m	m	m

phases (Fig. 1a). In three specimens (ZUEC 17925, ZUEC 12363 and MNRJ 24224), a size heteromorphism was observed between the homologous NORs by FISH with an rDNA probe (Fig. 1c) and by silver staining (Fig. 1c - inset). In two specimens (MNRJ 24230 and 24232), the NOR-bearing homologous chromosomes 8 were homomorphic. For the remaining specimens, we were not able to determine if a NOR size heteromorphism was present.

Physalaemus albonotatus

The *P. albonotatus* chromosomes were classified as metacentric (pairs 1, 2, 5, 6, 8, 9, 10 and 11), submetacentric (pairs 4 and 7) or subtelocentric (pair 3, which is at the threshold between submetacentric and subtelocentric classifications) (Fig. 2a; Table 2). Curiously, chromosome 5 was larger than chromosomes 3 and 4 in some of the analyzed metaphases (as seen in Figure 2b). Heterochromatin was detected in the centromeres of all chromosomes and interstitially in the long arm of chromosome 2 and in the metacentric chromosome 5 (Fig. 2b). Only two C-banded chromosome pairs 5 were good enough to be measured. Therefore, we tentatively assigned the interstitial C-band of chromosome 5 to its short arm, but further analyses are necessary to test this hypothesis. Chromosomes 3 and 4 were very similar, but chromosome 3 had a slightly smaller centromeric index and a strong centromeric C-band, which extended to the short arm (Fig. 2b - inset; Table 2).

Silver staining detected NORs distally in the long arm of chromosome 8 adjacent to a faint C-band and in both arms of chromosome 9 (Fig. 2d). The NOR in the long arm of chromosome 9 apparently coincided with a C-band (Fig. 2d). All of these NORs could be seen as secondary constrictions in Giemsa-stained metaphases (Figs. 2a and 2c).

Physalaemus centralis

The *P. centralis* chromosomes were classified as metacentric (pairs 1, 2, 5, 6, 8, 9, 10 and 11) or submetacentric (pairs 3, 4, 7 and 8) (Fig. 3a; Table 2). A secondary constriction was detected in the pericentromeric region of the long arm of chromosome 9 and coincided with the NOR that was recognized by silver staining (Fig. 3a - inset). A NOR size heteromorphism was observed in all of the *P. centralis* specimens analyzed. C-bands were present interstitially in the long arm of chromosome 2, in the short arm of chromosome 5, in the long arms of chromosomes 8 and 9, in the pericentromeric region of the short arm of chromosome 10, and in all of the centromeres (Fig. 3b). Chromosomes 3 and 4 were very similar, but chromosome 3 had a slightly smaller centromeric index and a strong centromeric C-band, which extended to the short arm (Fig. 3b; Table 2).

In three specimens, a heteromorphic chromosome pair 8 composed of homologues with different morphologies and C-banding patterns was observed (Figs. 3a and 3b). While one chromosome 8 showed a conspicuous interstitial C-band that sometimes could be seen as two heterochromatic blocks (chromosome 8a in Fig. 3b), its homologue had no observable interstitial heterochromatic block (Fig. 3b). In the ZUEC 13696 specimen, the pericentromeric C-bands in the long arms of the homologous chromosomes 2 were heteromorphic in size. Additionally, the homologue that had the smaller pericentromeric C-band also had an additional and conspicuous terminal C-band in the long arm (Fig. 3b - inset).

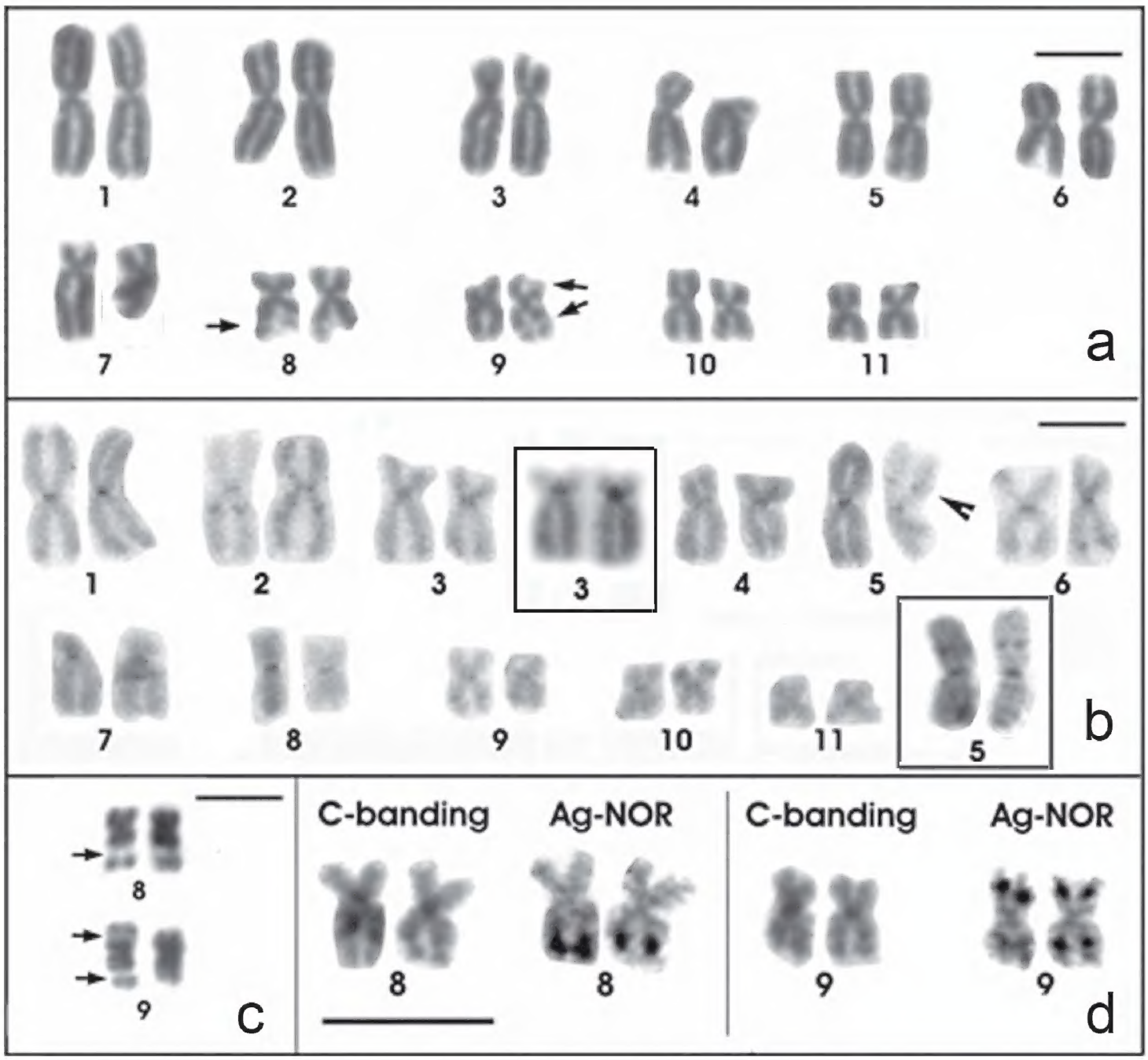

Figure 2. Giemsa-stained (**a**) and C-banded (**b**) karyotypes of *P. albonotatus*. In the insets in **b** C-banded chromosome pairs 3 and 5, showing evident pericentromeric and interstitial bands, respectively **c** NOR-bearing chromosome pairs of *P. albonotatus* stained with Giemsa. Arrows in **a** and **c** indicate secondary constrictions of the NORs. Arrowhead in **b** indicates the C-band in chromosome 5 **d** NOR-bearing chromosome pairs of one specimen of *P. albonotatus* sequentially submitted to the C-banding and the Ag-NOR methods. Note the NOR adjacent to an interstitial C-band in pair 8 and the NORs coincident with faint C-bands in pair 9. Bar=10mm.

Physalaemus cuqui

The *P. cuqui* chromosomes were classified as metacentric (pairs 1, 2, 5, 6, 8, 9, 10 and 11), submetacentric (pairs 4 and 7) or subtelocentric (pair 3, which is at the threshold between submetacentric and subtelocentric classifications) (Fig. 4a; Table 2). Heterochromatic bands were observed interstitially in the long arm of chromosome pair 2, in the metacentric chromosome pair 5 and in the centromeric regions of all of the chromosomes (Fig. 4b). Only one C-banded chromosome pair 5 could be measured. Therefore, as well as for *P. albonotatus*, we tentatively assigned the interstitial C-band of chromosome 5 of *P. cuqui* to its short arm, but further analyses are necessary to test

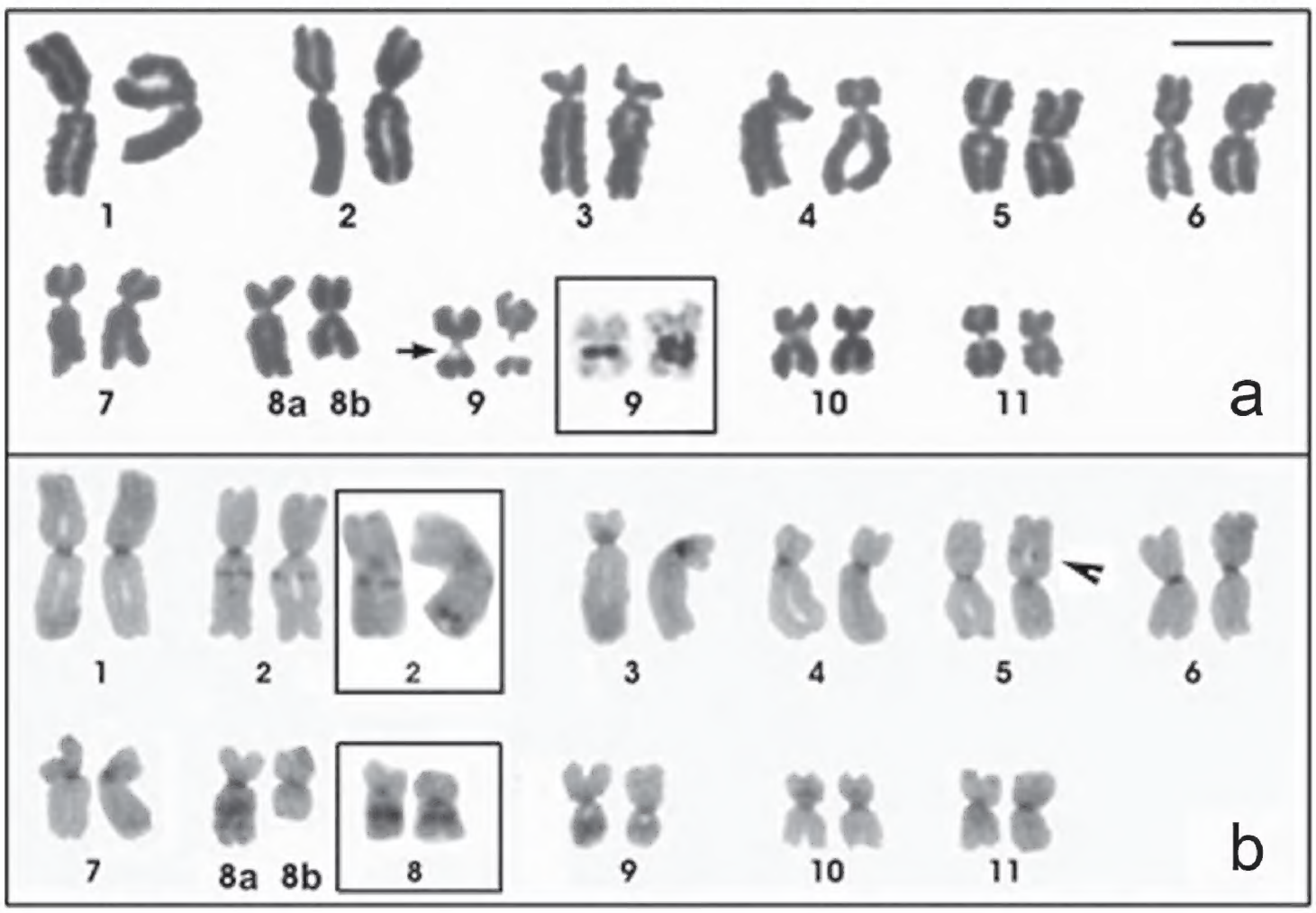

Figure 3. Giemsa-stained (**a**) and C-banded (**b**) karyotypes of *P. centralis*. In **a** an arrow indicates the secondary constriction of the NOR and the inset shows the NOR-bearing chromosome 9 after silver staining. Note the NOR size heteromorphism. In **b** the arrowhead indicates the C-band in 5p and the insets show the heteromorphic pair 2 and the homomorphic pair 8 of the ZUEC 13696 specimen. Note the conspicuous interstitial heterochromatin in the long arm of chromosome pair 8. Bar=10mm.

this hypothesis. Chromosomes 3 and 4 were very similar, but chromosome 3 had a slightly smaller centromeric index and a strong centromeric C-band, which extended to the short arm (Fig. 4b; Table 2).

In three specimens, the Ag-NORs were located in the long arm of chromosome pair 8 and in the short and long arms of chromosome pair 9 (LGE 1635-1636, MLP DB 4973) (Fig. 4c – left), but only one chromosome 9 was silver-stained in the MLP DB 5560 specimen (Fig. 4c – middle). Additionally, one specimen (MLP DB 6480) showed an additional Ag-NOR in the short arm of one chromosome 4 (Fig. 4c – right). These Ag-NORs were coincident with the secondary constrictions visualized in Giemsa-stained metaphases (Fig. 4a).

Physalaemus santafecinus

The *P. santafecinus* chromosomes were classified as metacentric (pairs 1, 2, 3, 5, 6, 8, 9, 10 and 11) or submetacentric (pairs 4 and 7) (Fig. 5a; Table 2). The NORs were located distally in the long arm of chromosome 9 (Fig. 5a - inset). C-bands were detected in all the centromeric regions. Additionally, pericentromeric C-bands were present in the short arms

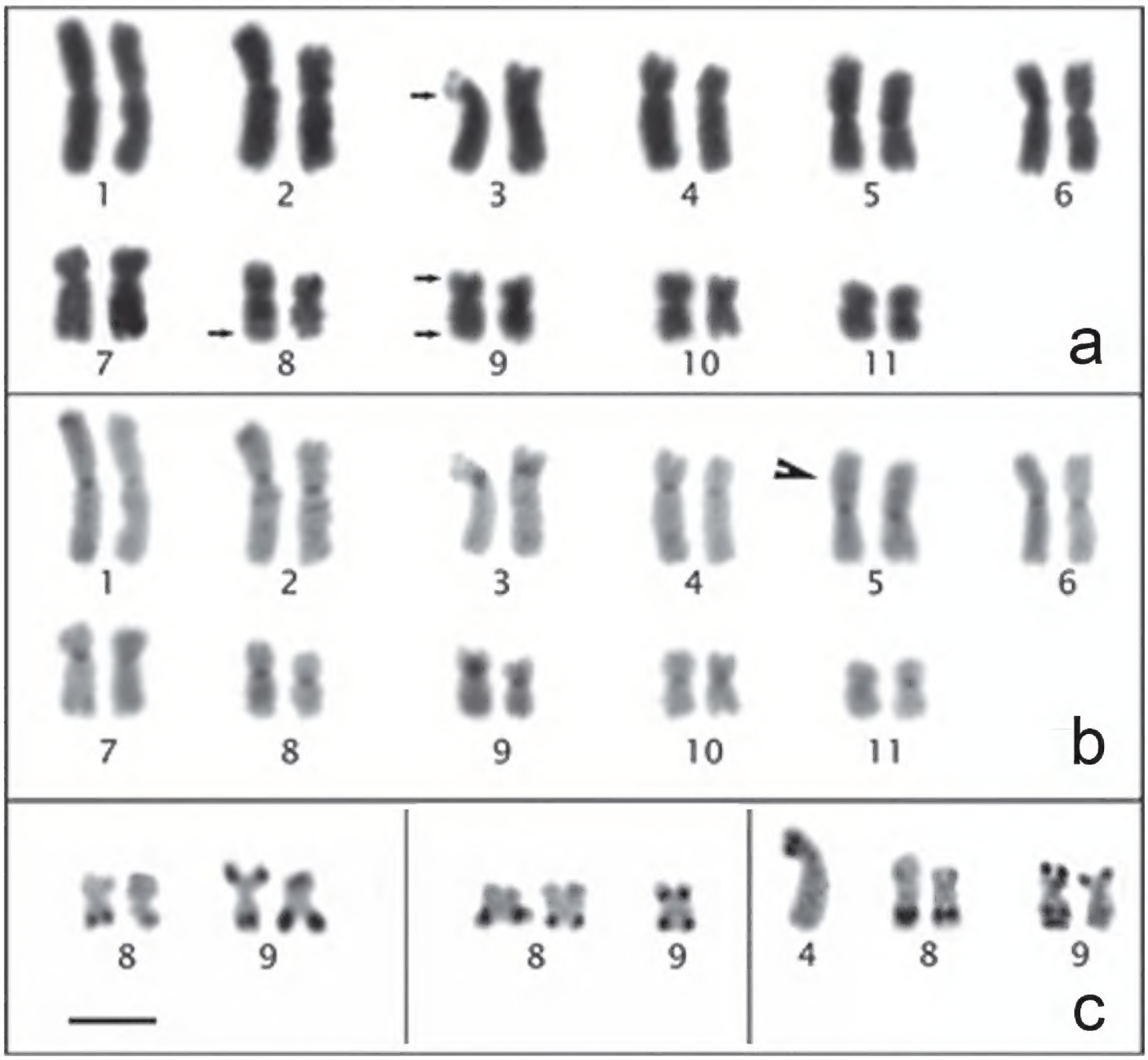

Figure 4. Giemsa-stained (**a**) and C-banded (**b**) karyotypes of *P. cuqui*. In **a** the arrows indicate the secondary constriction of the NOR. In **b** arrowhead indicates the C-band in 5p **c** Variability in the NOR-bearing chromosome pairs of three specimens of *P. cuqui.* Bar=10mm.

of chromosomes 1 and 2 and in the short arm of chromosome 8. Small C-bands were also detected proximally in the long arms of chromosomes 4 and 7 and distally in the long arm of chromosome 1. A conspicuous C-band was observed in the short arm of chromosome 3, which was almost entirely heterochromatic. Terminal faint C-bands could be seen in all of the chromosomes (Fig. 5b). When the Ag-NOR method was performed on C-banded metaphases, we could undoubtedly recognize the chromosome 9 as the NOR-bearing chromosome while chromosomes 8 had strong pericentromeric C-bands (data not shown).

Discussion

To date, 23 of the 46 species of *Physalaemus* were karyotyped and all of them have 2n=22 (Beçak 1968, Beçak et al. 1970, Denaro 1972, De Lucca et al. 1974, Silva

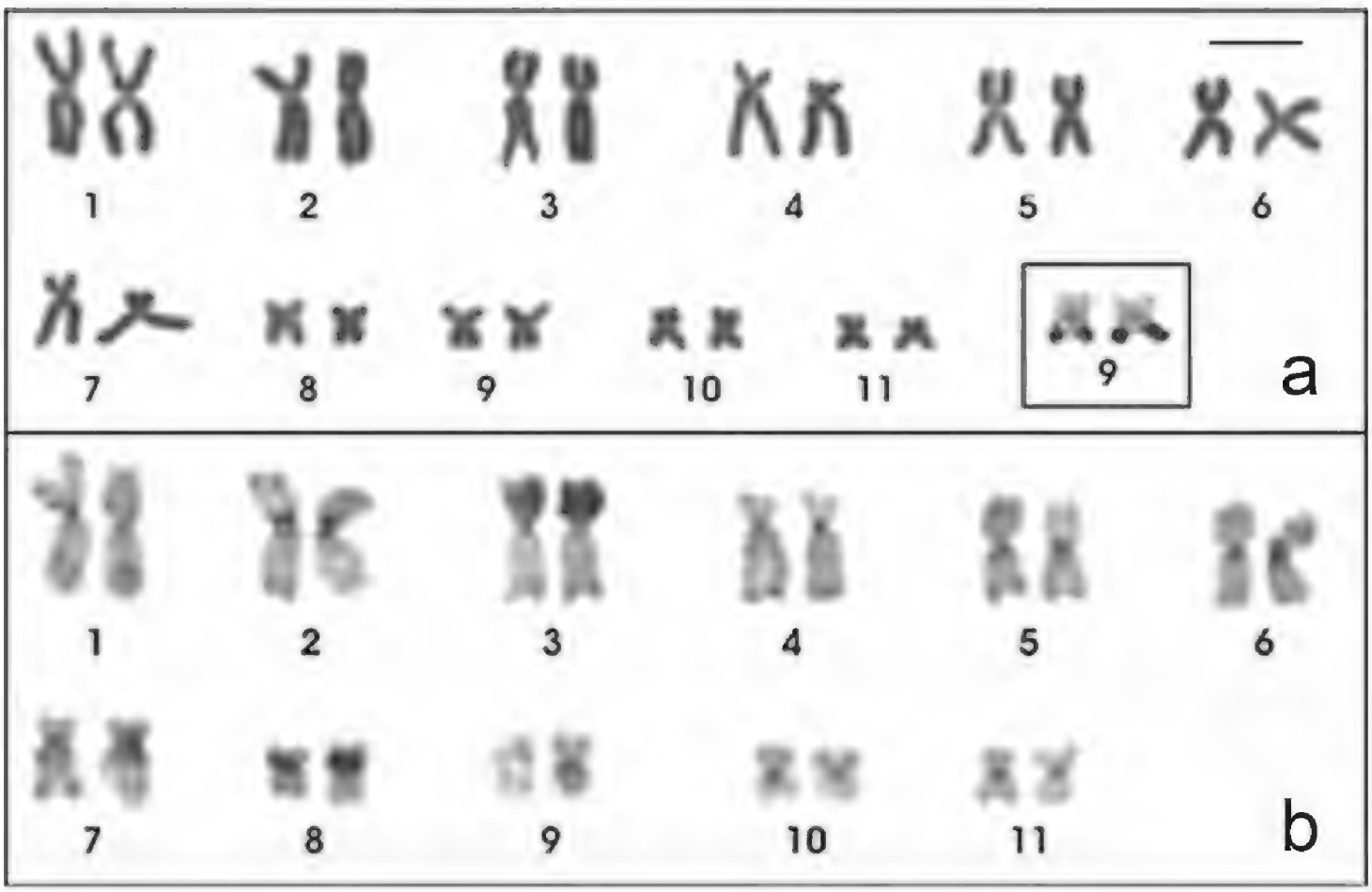

Figure 5. Giemsa-stained (**a**) and C-banded (**b**) karyotypes of *P. santafecinus*. In **a**, the inset shows the NOR-bearing chromosome 9 after silver staining. Bar=10mm.

et al. 1999, Silva et al. 2000, Amaral et al. 2000, Lourenço et al. 2006, Ananias et al. 2007, Tomatis et al. 2009, Milani et al. 2010 – included *P. feioi* Cassini et al., 2010 as *P. olfersii* (Lichtenstein & Martens, 1856), Nascimento et al. 2010, Provete et al. 2012). Interestingly, two distinct fundamental numbers (FN) can be recognized among the karyotypes of *Physalaemus* species. The five species of the *P. signifer* group already karyotyped have FN=42 and a telocentric chromosome 11 [see karyotype of *P. signifer* (Girard, 1853) in De Lucca et al. (1974), *P. crombiei* Heyer & Wolf, 1989 and *P. spiniger* (Miranda-Ribeiro, 1926) karyotypes in Silva et al. (2000), and a reference to the *P. atlanticus* Haddad and Sazima, 2004 and *P. moreirae* (Miranda-Ribeiro, 1937) karyotypes in the discussion of Ananias et al. (2007)], as does *P. nattereri* (Beçak 1968, Lourenço et al. 2006, Ananias et al. 2007) and *P. fernandezae* (Müller, 1926) (Tomatis et al. 2009). The karyotypes of the remaining species of *Physalaemus*, including the species of the *P. cuvieri* and the *P. albifrons* groups that we focused on in our present investigation, have FN=44 and a biarmed chromosome 11. Considering the close phylogenetic relationship inferred for *P. nattereri* and *P. signifer* (Pyron and Wiens 2011, Faivovich et al. 2012, Fouquet et al. 2013), which was the only species of the *P. signifer* group already included in phylogenetic analyses, it is possible to suppose that the telocentric chromosomes 11 of *P. nattereri* and *P. signifer* have the same origin. On the contrary, the similar chromosomes 11 of *P. fernandezae* and the *P. signifer* group probably result from a homoplasy (Tomatis et al. 2009).

The karyotype of *P. santafecinus* described here is very similar in chromosomal size and morphology to those of *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* (Amaral et al. 2000, Silva et al. 2000). The chromosomes classified by Amaral et al. (2000) as 4 and 5 probably correspond to chromosomes 5 and 4, respectively, of the karyotype of *P. biligonigerus* described by Silva et al. (2000) and of the *P. santafecinus* karyotype. Such a discrepancy emerges, however, from the use of different criteria for the numeric classification of the chromosomes rather than from a real divergence between the karyotypes.

A remarkable characteristic of the *P. santafecinus* karyotype that is shared with the karyotypes of *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* is a conspicuous C-block on the short arm of chromosome 3 (3p) (Table 3). This large heterochromatic C-block is not detected in *P. albifrons* or in any species of *P. cuvieri* group. Instead, a small C-band pericentromerically located on 3p was already detected in the karyotypes of the species currently allocated to the *P. cuvieri* group that were already studied by C-banding [i.e., *P. albifrons*, *P. albonotatus*, *P. centralis*, *P. cuqui* (present work), *P. ephippifer* (Steindachner, 1864) (Nascimento et al. 2010) and one of the populations of *P. cuvieri* Fitzinger, 1826 that was studied cytogenetically by Quinderé et al. (2009)]. Although the pericentromeric C-band in 3p of *P. ephippifer* could be easily observed, it was also much smaller than those observed in *P. santafecinus*, *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus.* In the latter four species, the larger size of this C-band probably explains the larger size of 3p in these karyotypes. A small pericentromeric C-band that extend from the centromere to the short arm of chromosome 3 was also present in *P. barrioi* Bokermann, 1967 (Provete et al., 2012), *P. olfersii* and *P. feioi* (as *P. olfersii*; Milani et al. 2010), which are the species of *P. gracilis* group (*P. barrioi*) and *P. olfersii* group (*P. olfersii* and *P. feioi*) already studied by C-banding.

Interestingly, a large 3p showing a large C-band was also observed in *P. nattereri* (Lourenço et al. 2006, Ananias et al. 2007), a species previously allocated to the *P. biligonigerus* group by Lynch (1970). Although a rigorous phylogenetic analysis of the *Physalaemus* genus is not yet available, in recent phylogenetic inferences *P. nattereri* was recovered as the sister species of *P. signifer* and was not closely related to *P. biligonigerus* (Pyron and Wiens 2011, Faivovich et al. 2012, Fouquet et al. 2013). In this phylogenetic context the most parsimonious hypothesis is to consider the large heterochromatic region in chromosome 3 of *P. nattereri* to be homoplastic with respect to the large heterochromatic region in chromosome 3 of the *P. santafecinus*, *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* karyotypes. This hypothesis is particularly plausible if we consider the evolutionary dynamics of satellite DNAs, which are the principal components of heterochromatin (reviewed in Charlesworth et al. 1994). The copy number of satellite DNA repeats can vary dramatically, as they are frequently involved in unequal crossing over and other events as rolling circle replication and conversion-like mechanisms (reviewed in Charlesworth et al. 1994, and in Ugarkovic and Plohl 2002).

On the other hand, the available data do not prevent the large C-band found on 3p of *P. santafecinus*, *P. biligonigerus* and *P. marmoratus* from being a synapomorphy of this group of species, which could have arisen from the amplification of a small C-band.

Table 3. Comparison of chromosome 3 of species of *P. cuvieri* (left column) and P. *albifrons* (right column) groups. Black areas in the ideograms represent C-bands. *[1]Based on Silva et al. (1999) and Quinderé et al. (2009). *[2]Nascimento et al. (2010). *[3]Based on Amaral et al. (2000).

***P. cuvieri* group (sensu Nascimento et al. 2005)**	**Chromosome 3**		***P. albifrons* group (sensu Nascimento et al. 2005)**
P. albonotatus			*P. albifrons*
P. centralis		*3	*P. biligonigerus*
P. cicada	No C-banding data	*3	*P. marmoratus (=P. fuscomaculatus)*
P. cuqui			*P. santafecinus*
P. cuvieri	*1		
P. ephippifer	*2		
P. erikae	No C-banding data		
P. fischeri	No C-banding data		
P. kroyeri	No C-banding data		

Despite the proposals of Lynch (1970) and Nascimento et al. (2005) disagree with regard to the relationships of these three species with other *Physalaemus* species, the close relationships of *P. santafecinus*, *P. biligonigerus* and *P. marmoratus* was considered in both studies. A phylogenetic analysis designed to study the relationships in the genus *Physalaemus*, however, is crucial to test this hypothesis. Also, further molecular characterization of the heterochromatic bands on 3p could help to provide additional evidence of the inferred heterochromatin amplification process.

In addition to the large C-band in 3p, the karyotype of *P. santafecinus* is also similar to those of *P. biligonigerus P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* (Amaral et al. 2000, Silva et al. 2000) based on the presence of several telomeric C-bands and a pericentromeric C-band in the short arm of chromosome 8 as well as the NOR location. In all of these karyotypes, the NOR-bearing chromosome is small and metacentric, and it was classified as chromosome 9 in the karyotype of *P. santafecinus* (described here) and in the karyotypes of *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus* described by Amaral et al. (2000). However, in the karyotype of *P. biligonigerus* described by Silva et al. (2000), the NOR-bearing chromosome was considered to be chromosome 8, which has a conspicuous pericentromeric C-band. Because Silva et al. (2000) apparently did not perform sequential C-banding and Ag-NOR in order to properly identify the NOR-bearing chromosome in C-banded metaphases, it is likely that the NOR-bearing chromosome is chromosome 9 in the C-banded karyotype shown by those authors.

In contrast to *P. santafecinus*, *P. biligonigerus*, *P. marmoratus* and *Physalaemus* sp. aff. *biligonigerus*, the telomeric C-bands could not be detected in the karyotype of *P. albifrons*. Additionally, the NOR in *P. albifrons* was detected interstitially in the long arm of the submetacentric chromosome 8. This NOR-bearing chromosome very closely resembles the NOR-bearing chromosome found in some populations of *P. cuvieri* (Silva et al. 1999, Quinderé et al. 2009) as well as in *P. albonotatus* and *P. cuqui* (present work). The *P. albifrons* karyotype presented here is very similar to the Giemsa-stained karyotype described for this species by Denaro (1972). However, the chromosome classified by Denaro (1972) as No. 11 is probably the one we classified as No. 8, and the secondary constriction observed by Denaro (1972) is likely to be the site recognized as NOR by silver impregnation in the present work.

Despite the similarity between the NOR-bearing chromosome of *P. albifrons* and those of some species of the *P. cuvieri* group, it would be premature to consider this a synapomorphy of *P. albifrons* and species of the *P. cuvieri* group because the evolutionary divergence of this character (i.e., NOR location) has not yet been elucidated. We cannot discard the possibility that the NOR found in *P. albifrons* and in some *P. cuvieri* species is plesiomorphic with respect to the other NOR sites found in *Physalaemus* species. This interpretation derives from the fact that the NOR-bearing chromosome 8 found in other leiuperines, as *Pleurodema diplolister* (Peters, 1870) (Lourenço et al., 2006), resembles that of *P. albifrons* and some *P. cuvieri* species group and could constitute the same state of character.

Another chromosome feature found in *P. albifrons* that was also detected in species of the *P. cuvieri* group was the interstitial C-band in chromosome 5 (Table 4). This C-

Table 4. Comparison of chromosome 5 of species of *P. cuvieri* (left column) and *P. albifrons* (right column) groups. Black areas in the ideograms represent C-bands. *[1]C-band was tentatively assigned to the short arm (see text for details). *[2]Based on Silva et al. (1999) and Quinderé et al. (2009). *[3]Nascimento et al. (2010). *[4]Based on chromosomes described as No. 3 by Amaral et al. (2000).

***P. cuvieri* group (sensu Nascimento et al. 2005)**	**Chromosome 5**		***P. albifrons* group (sensu Nascimento et al. 2005)**
P. albonotatus	*1		*P. albifrons*
P. centralis		*4	*P. biligonigerus*
P. cicada	No C-banding data	*4	*P. marmoratus (=P. fuscomaculatus)*
P. cuqui			*P. santafecinus*
P. cuvieri	*2		
P. ephippifer	*3		
P. erikae	No C-banding data		
P. fischeri	No C-banding data		
P. kroyeri	No C-banding data		

band was observed in all of the species of the *P. cuvieri* group already analyzed by the C-banding technique, including *P. cuvieri* (Silva et al. 1999, Quinderé et al. 2009), *P. ephippifer* (Nascimento et al. 2010), *P. albonotatus* (present work), *P. centralis* (present work) and *P. cuqui* (present work). However, this band was not detected in the C-banded karyotypes of the other three species currently allocated in the *P. albifrons* group (Amaral et al. 2000, Silva et al. 2000, present work) or in species of the *P. henselii* group (Tomatis et al. 2009), the *P. olfersii* group (Milani et al. 2010) and the *P. gracilis* group (Provete et al. 2012). Based on these data, the interstitial C-band in the medium-sized chromosome classified as No. 5 is a putative synapomorphy of *P. albifrons* and the species of the *P. cuvieri* group. However, because of the small size of this C-band, which could make its detection by the C-banding technique particularly difficult, and because of the dynamics of the satellite DNA sequences, which are subject to recurrent amplification/deletion events, this hypothesis must be taken with caution. A comprehensive phylogenetic study of the genus *Physalaemus* and a molecular characterization of this interstitial C-band would allow this hypothesis to be properly evaluated.

In conclusion, we were not able to recognize any chromosomal character that would support the reallocation of *P. albifrons* from the *P. cuvieri* group to the *P. albifrons* group together with *P. biligonigerus*, P. *marmoratus* and *P. santafecinus*.

Interestingly, in addition to the data regarding chromosomal characteristics, larval morphology also does not seem to support the composition of the *P. albifrons* group. *Physalaemus biligonigerus*, *P. santafecinus* and *P. marmoratus* have a similar larval oral disc configuration (LTRF 2/2, with a dorsal gap in the marginal papillae) that differs considerably from that of *P. albifrons*, whose oral disc is almost identical to that of the tadpoles of the *P. cuvieri* group and is thus characterized by an LTRF 2/3 with dorsal, ventrolateral and ventral gaps in the marginal papillae (Vera Candioti et al. 2011). During embryogenesis of the oral disc of *Physalaemus*, ventrolateral gaps appear in the marginal papillae, apparently in all species of the genus (see Vera Candioti et al. 2011). The ventrolateral gaps persist only in the tadpoles of *P. cuvieri* species group [except *P. fischeri* (Boulenger, 1890) and *P. cicada* Bokermann, 1966], in *P. riograndensis* Milstead, 1960 (*P. henselii* group) and in *P. albifrons* (see Vera Candioti et al. 2011). On the other hand, ventral gaps develop only in tadpoles of *P. albifrons*, in species of *P. cuvieri* group (except *P. fischeri*) and in two species of the *P. henselii* group [*P. henselii* (Peters, 1872) and *P. fernandezae* (Müller, 1926)]. Among the leiuperines, the ventrolateral gaps were only observed in some species of *Pseudopaludicola* (see Vera Candioti et al. 2011), and although its presence during development appears to be plesiomorphic for *Physalaemus*, its persistence in larval stages is a putative synapomorphy of the *P. cuvieri* group (including *P. albifrons*). Finally, the internal oral morphology of tadpoles of *P. albifrons* differs from that of *P. biligonigerus*, *P. marmoratus* and *P. santafecinus* based on the presence of three lingual papillae, which is a characteristic shared with some species of the *P. cuvieri* group (Oliveira et al. 2010).

Interspecific comparison in the *P. cuvieri* group

Some of the species in the *P. cuvieri* group are sibling species with important intraspecific morphological variation. Therefore, the identification of these species that is based exclusively on their morphology is sometimes very difficult. Occasionally, species misidentification has occurred, for example, among *P. cuvieri*, *P. albonotatus*, *P. cuqui* and *P. centralis* (Barrio, 1965). Our results revealed conspicuous cytogenetic differences among most species of the *P. cuvieri* group. The exception is the great similarity between the karyotypes of *P. albonotatus* and *P. cuqui*. Additionally, the karyotypes of the species analyzed here were distinguished from the previously analyzed karyotype of *P. cuvieri*. The interspecific variation described in this work regarding heterochromatin and NOR distribution is of fundamental importance for the comparative analysis of the *P. cuvieri* species group.

An interstitial C-band was observed near the centromere in the long arm of chromosome 2 of *P. albonotatus*, *P. centralis* and *P. cuqui*; whereas in the karyotype of *P. ephippifer* (Nascimento et al. 2010) there is an interstitial C-band in the short arm of chromosome 2. A corresponding interstitial C-band in the short arm of chromosome 2 was reported in *P. cuvieri* populations from Rio Claro (Silva et al. 1999) and Palmeiras (Quinderé et al. 2009). If these heterochromatic bands were homeologous, it is conceivable that rearrangements (mainly pericentric inversions) involving chromosome 2 might have occurred during the divergence of these species. Interestingly, the present work reports evidence of a rearrangement involving chromosome 2 in *P. centralis*. In the ZUEC 13696 specimen of *P. centralis*, heteromorphism for the intrachromosomal location of heterochromatic regions in the chromosome pair 2 suggested that paracentric inversion might have been involved in this chromosomal rearrangement.

Despite the overall similarity in chromosomal morphology among the species currently allocated to the *P. cuvieri* group, chromosome pairs 8 and 9 differ greatly. The differences in these chromosomes probably arose from the distinct locations of the NOR in these karyotypes, as these rDNA genes occupy different sites in pairs 8 and/or 9 of these species. The observed pattern of NOR occurrence can be helpful in distinguishing the analyzed species of the *P. cuvieri* group. Noticeably, a pericentromeric NOR site was found exclusively in the *P. centralis* karyotype. However, the NOR-bearing chromosomes (chromosome pairs 8) from the species *P. cuvieri* (Silva et al. 1999, Quinderé et al. 2009), *P. albonotatus*, *P. cuqui* and *P. albifrons* are quite similar and their homeology could be possible. Otherwise, the evolutionary relationship of this chromosome with the other NOR-bearing chromosomes found in species of *P. cuvieri*, *P. albifrons* and other species groups remains unclear, and further studies are necessary to elucidate the rearrangements that give rise to the great diversification of the NOR-bearing chromosomes in this genus.

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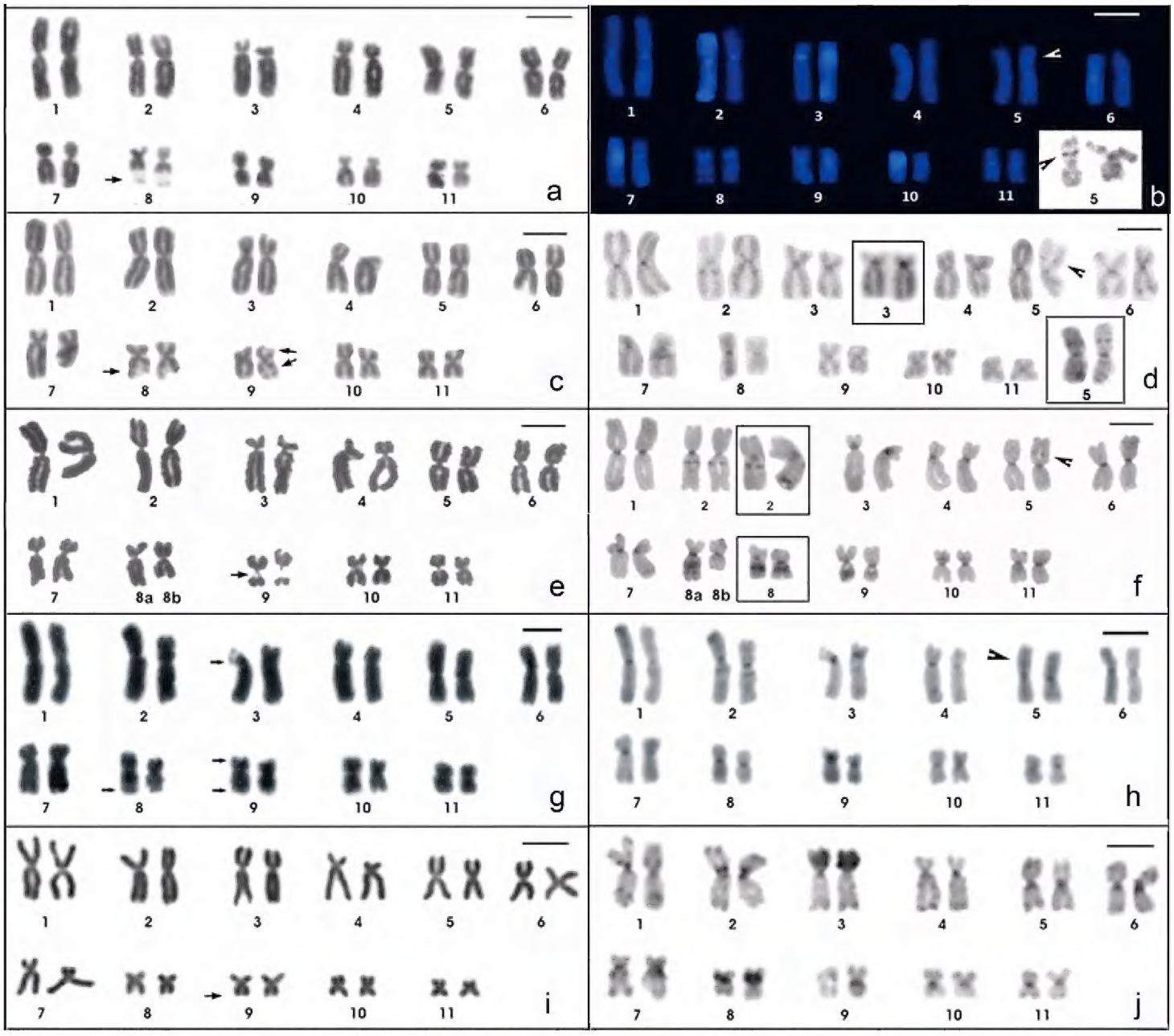
Appendix



Additional file 1. Giemsa-stained (**a, c, e, g, i**) and C-banded karyotypes of *P. albifrons* (**a, b**) *P. albonotatus* (**c, d**) *P. centralis* (**e, f**) *P. cuqui* (**g, h**) and *P. santafecinus* (**i, j**). In **b** the chromosomes were stained with DAPI after C-banding, except those in the inset, which were stained with Giemsa. In the insets in **d** C-banded chromosome pairs 3 and 5, showing evident pericentromeric and interstitial bands, respectively. The insets in **f** show the heteromorphic pair 2 and the homomorphic pair 8 of the ZUEC 13696 specimen. Arrows point NORs. Arrowheads point the C-band in 5p. Bar=10mm.